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REACTION MECHANISM
OF
BENZYLAMINE OXIDASE

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BENZYLAMINE OXIDASE

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by

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PUBLICATIONS

This thesis is based on the following papers, which will be referred to by Roman numerals in the text.

- I. Transient Kinetics of Benzaldehyde Formation during the Catalytic Action of Pig-Plasma Benzylamine Oxidase.
Anders Lindström, Bengt Olsson, and Gösta Pettersson (1974)
Eur. J. Biochem. 42, 377-381.
- II. Kinetics of the Interaction between Pig-Plasma Benzylamine Oxidase and Various Monoamines.
Anders Lindström, Bengt Olsson, Gösta Pettersson, and Jadwiga Szymanska (1974)
Eur. J. Biochem. 47, 99-105.
- III. Kinetics of the Interaction between Pig-Plasma Benzylamine Oxidase and Hydrazine Derivatives.
Anders Lindström, Bengt Olsson, and Gösta Pettersson (1974).
Eur. J. Biochem. 42, 177-182.
- IV. Effect of pH on the Transient Reduction of Pig-Plasma Benzylamine Oxidase by Benzylamine Derivatives.
Anders Lindström, Bengt Olsson, Jadwiga Olsson, and Gösta Pettersson (1976).
Eur. J. Biochem. 64, 321-326.
- V. Kinetic Isotope Effects on the Catalytic Activity of Pig-Plasma Benzylamine Oxidase.
Bengt Olsson, Jadwiga Olsson, and Gösta Pettersson (1976).
Eur. J. Biochem. 64, 327-331.
- VI. Stopped-Flow Spectrophotometric Characterization of Enzymatic Reaction Intermediates in the Anaerobic Reduction of Pig-Plasma Benzylamine Oxidase by Amine Substrates.
Bengt Olsson, Jadwiga Olsson, and Gösta Pettersson.
Eur. J. Biochem. (Submitted for publication).
- VII. The Kinetics of Reoxidation of Reduced Benzylamine Oxidase.
Bengt Olsson, Jadwiga Olsson, and Gösta Pettersson.
Eur. J. Biochem. (Submitted for publication).

INTRODUCTION

The metabolism of a living organism is a complicated interlocking pattern of sequential chemical reactions, and with very few exceptions each separate metabolic step is catalysed by an enzyme. An enzyme may be defined as "a protein with catalytic properties due to its power of specific activation". The amine oxidases represent a group of enzymes of particular biological interest as they act on biogenic amines which are involved in vital regulatory mechanisms. Amine oxidases oxidatively deaminate amines with stoichiometric formation of one molecule each of aldehyde, ammonia and hydrogen peroxide.



Classification of amine oxidases

Zeller early classified the amine oxidases into two groups; the monoamine oxidases and the diamine oxidases (1). This classification was based on supposed substrate specificities of these enzymes, and was recommended by the International Union of Biochemistry. Later, however, more extensive studies revealed that some monoamine oxidases oxidize long-chain aliphatic diamines which are not degraded by diamine oxidases (2). These and other observations led to a classification based on the inhibitor specificity:

- 1) Amine oxidases which are unaffected by carbonyl reagents.
- 2) Amine oxidases which are inhibited by carbonyl reagents.

The first group includes the FAD-containing monoamine oxidases located mainly in the outer membrane of the mitochondrion (3). The physico-logical role of these enzymes in the biological inactivation of catecholamines at nerve endings has been well established (4). It is now generally agreed that metals are not cofactors for mitochondrial monoamine oxidase catalysis.

However, enzymes belonging to the second group of amine oxidases all seem to contain Cu. Highly purified preparations of Cu-containing amine oxidases have been obtained from the fungus *Aspergillus niger*, pea seedlings, bovine blood plasma, pig kidney cortex and pig plasma (5). Some basic physico-chemical data for these enzymes are summarized in Table 1. The Cu-containing amine oxidases have many properties in common but also differ in several respects particularly in the substrate specificity exhibited. Amine oxidases from ruminant-plasma, for instance, oxidase the polyamine spermine even though non-ruminant enzymes do not (6). In contrast to the mitochondrial

amine oxidases, the Cu-containing amine oxidases act only on primary amines.

Source	Mol. weight	Cu-content		Carbonyl groups	
pea seedlings	96 000 (7)	1	(12)	1	(17)
pig plasma	195 000 (8)	2	(13)	1 (18)	3 (19)
beef plasma	170 000 (9)	2	(14)	1	(20)
<i>Aspergillus</i>					
Niger	252 000 (10)	3	(15)	2	(21)
pig kidneys	185 000 (11)	2	(16)		

Table 1

Cu-containing amine oxidases

Copper is the only metal which has been detected in significant amounts in the amine oxidases listed in Table 1. The copper content reported for these enzymes appears to be consistent with the presence of one copper atom per subunit. In several cases it has been possible to remove the metal ions by treating the enzymes with diethyl dithiocarbamate. The activity is lost but reactivation is achieved by adding Cu^{2+} (12, 14-16, 22, 23).

One of the most useful spectroscopic tools in the study of copper-containing proteins is electron paramagnetic resonance (E.P.R.) (24), which can be applied to systems having unpaired electrons. As Cu^{2+} is paramagnetic but Cu^{1+} and Cu^{3+} have no unpaired electrons it is only the first ionic species which can be detected and studied by E.P.R. techniques. The content of E.P.R. detectable copper has been found to be less than the chemically determined amount in some amine oxidases (16,22, 25, 26).

In benzylamine oxidase, however, the amount of copper determines by integrating the E.P.R.-spectra agrees with the value obtained by atomic absorption spectroscopy and chemical analysis (13, 18, 27) and it appears to be well established that the two Cu-ions in benzylamine oxidase are both present in the cupric state.

The E.P.R.-spectra for all Cu-containing amine oxidases exhibit an axial symmetry and magnitudes of the E.P.R.-parameters for the Cu-signals, agree with these observed for planar tetragonal complexes with ligands of low molecular weight (16, 25, 27). The E.P.R. spectral properties of Cu in amine oxidases thus resemble those of type 2 Cu in blue oxidases (28, 29).

Several superhyperfine lines with a splitting of about 14 G in the $g_{\perp}$ region were detected in E.P.R.-spectra of amine oxidase from pig kidney, indicating that nitrogen ligands are involved in the binding of copper (16). A similar superhyperfine pattern is present in E.P.R.-spectra of benzylamine oxidase (30).

Measurements of the proton relaxation enhancement caused by benzylamine oxidase led to the conclusion (27) that water coordinated axially to the Cu^{2+} ions is rapidly exchanging with water in the bulk aqueous phase.

Examination of the effects of azide on spectral and catalytic properties of benzylamine oxidase support the idea that enzyme-bound copper is assessable for interaction with ligands in solution and provides evidence that at least one of the two copper atoms present has a catalytic function (31).

While x-band E.P.R.-spectra of benzylamine oxidase do not distinguish clearly between the Cu-atoms, Q-band spectra exhibit obvious contributions from two different signals of approximately equal intensity, which might suggest that the two Cu-atoms are in different chemical environments (27). In general, there is no significant reduction of the intensity of the E.P.R.-signal of Cu-containing enzymes, when amines are added under anaerobic conditions but minor changes in the shape of the spectra have been reported (13, 22, 25, 32, 33). Examination of the changes caused by ^{14}N -putrescine and ^{15}N -putrescine seem to exclude the possibility that the amine nitrogen of the substrate is a ligand to Cu^{2+} in the pig-kidney enzyme (16). With the latter enzyme, a slow reduction of the Cu-signal has been reported to occur on the anaerobic addition of substrates (34), but it is uncertain whether this reduction is of catalytic significance or not.

The reactivity towards carbonyl reagents of the Cu-containing amine oxidases is due to the presence of a prosthetic group, which in the oxidases from *Aspergillus Niger*, pig plasma and pig kidney has been tentatively identified as pyridoxal phosphate (PLP) (35, 36, 37). Attempts to establish the presence of PLP in the enzymes from bovine plasma and pea seedlings have, however, been unsuccessful (32, 38, 39). For the bovine enzyme it has been proposed that a sulphenic acid residue at the active site functions as an oxidizing agent (40). The presence of a -S-S group functioning as an electron acceptor

at the active site has also been proposed (41).

Attempts to establish the precise functional role of the prosthetic group and the metal in Cu-containing amine oxidases have been hampered by the lack of detailed knowledge about the basic reaction mechanism for these enzymes. In particular there has been no general agreement about the order of addition and release of substrates and products during catalysis. The significance and properties of enzyme intermediates formed in the catalytic process have been largely unknown. The present investigation summarizes the results obtained in a series of investigations aiming at providing such basic mechanistic information about the reactions catalyzed by the amine oxidase isolated from pig plasma, usually referred to as benzylamine oxidase.

Ping-Pong mechanism

Steady-State kinetic investigations of benzylamine oxidase have shown that the enzymatic reaction between amine substrates and oxygen adheres to the rate equation

$$\frac{V}{CE} = \phi_0 + \phi_1/[O_2] + \phi_2/[S]$$

where V/CE stands for the molar reaction velocity. The absence of a $\phi_{12}/[O_2][S]$ term might indicate that the reaction proceeds by a ping-pong mechanism, i.e. that the interaction between enzyme and one substrate leads to the release of the product and the formation of a modified enzyme species. Evidence for it has been noticed during the anaerobic interaction between enzyme and amine substrates (33). Formation of the modified enzyme can be followed spectrophotometrically as it leads to rapid absorbance changes in the visible region due to the reduction of a single chromophore centered around 470 nm. The rate of decolorization of this chromophore is unaffected by the concentration of oxygen but exhibits a dependence upon the substrate concentration, corresponding to a rate-determining bimolecular interaction between enzyme and substrate (33). Comparison between second-order rate constants for the decolorization process and steady-state kinetic parameters has established that reduction of the 470 nm chromophore reflects a catalytically significant process.

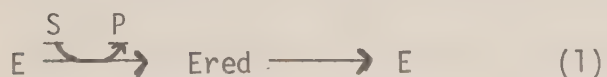
The main characteristic of a Ping-Pong mechanism is that after the addition of the first substrate at least one product must be released from the enzyme before the next substrate binds. In the case of the reaction between benzylamine oxidase and amine substrate this means that either hydrogen peroxide, ammonia or the aldehyde product must be formed before oxygen binds to the enzyme. Taylor et al (42) have found that ammonia is released from

this enzyme on the addition of benzylamine under highly anaerobic conditions. The other enzymes in Table I have, however, been reported to produce aldehyde when substrate is added in the absence of oxygen (17, 21, 43-45).

We have followed the formation of benzaldehyde during the catalytic action of benzylamine oxidase on benzylamine by stopped-flow techniques (paper I). In contrast to the results of Taylor et al, we found that the aldehyde product is formed prior to the interaction between enzyme and oxygen. Formation of aldehyde was found to occur in a rapid transient burst, reflecting a second order interaction between enzyme and substrate with a rateconstant of $1,2 \text{ s}^{-1} \text{ mM}^{-1}$. This value agrees with the second order rate constant for the anaerobic reduction of benzylamine oxidase by substrate as determined from the rate of decolorization of the 470 nm chromophore of the enzyme. This result indicates that the process of benzaldehyde formation cannot be kinetically distinguished from the process leading to the reduction of the 470 nm chromophore.

The amplitude of the burst was found to approach a saturation value approximating one mole of benzaldehyde formed per mole of enzyme at saturating substrate concentrations. This is consistent with the report that only one reactive pyridoxal phosphate is present per enzyme molecule (18).

The above results provide direct-evidence that benzylamine oxidase operates by a ping-pong mechanism involving two partial processes.



The first process includes the sequence of reactions occurring during the anaerobic interaction between enzyme and benzylamine. This partial process results in the release of the reaction product benzaldehyde and the formation of a modified enzyme species (Ered) which must be in a reduced state compared to the free enzyme. The second process includes reaction steps initiated by the interaction between reduced enzyme and oxygen, and leads to restoration of the enzyme.

Scheme (1) predicts that the same enzyme intermediate Ered is formed with all amine substrates. (This follows from the fact that the aldehyde product contains the entire structurally varied portion of different amine substrates.) Consequently, the reoxidation process, which is rate-limiting for the catalytic reaction during steady-state, should be identical for all amine substrates. These predictions are confirmed for different substrates in paper II, which says that maximum activities do not differ significantly from the value of $0,15 \text{ s}^{-1}$ determined for benzylamine. The magnitude of K_m varies over a

large range with different substrates and increases with the base strengthening of the amine. There is an approximately linear correlation between $\log K_m$ and pK_a . The second-order rate constants for reduction by various substrates are inversely proportional to the respective K_m -values, confirming that formation of the enzyme species lacking the 470 nm chromophore reflects the catalytically significant reduction of the enzyme by substrate.

Hydrazines as Substrate Analogues

Reduction of the enzyme can be anticipated as involving several reaction steps, e.g. binding the amine substrate to the enzyme, interaction between the substrate and prosthetic group of the enzyme and the hydrolytic release of the aldehyde product. Since it seemed impossible to distinguish these steps by stopped-flow techniques a simpler model for the binding of substrates to the enzyme was attempted.

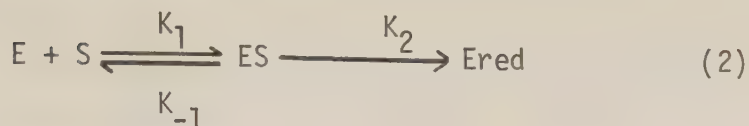
Phenylhydrazine has been shown to inactivate the enzyme irreversibly by binding to the prosthetic group of the enzyme (18). In paper III different hydrazines are tested as models for the interaction between enzyme and substrate. The interaction between enzyme and hydrazine derivatives can be followed by stopped-flow techniques at wavelengths in the 390-430 nm region, where the inactive enzyme complexes formed exhibit strong absorption. Examination of the effect of substrates on the kinetics of interaction between enzyme and hydrazines led to the conclusion that substrate and inhibitor compete for the prosthetic group of the enzyme. Formation of the enzyme - hydrazine complexes may therefore be expected to provide information about the binding of substrate to the prosthetic group of the enzyme. The pH-studies carried out provided evidence that the non-protonated form of the hydrazine derivatives is the reactive species. Furthermore, protonation of an ionizing group in the enzyme (pK_a 8.8) is required for a reaction to take place.

Effects of pH on the reduction process

The results obtained with the hydrazine derivatives suggested that investigation of the pH-dependence of the interaction between enzyme and substrate might provide additional information about the reduction process. The results of such an investigation are discussed in paper IV.

At pH 7 the rate of decolorization of the 470 nm chromophore by benzylamine is approximately linearly dependent on substrate concentration. At alkaline pH, however, deviations from a linear relationship become evident, and the rate constant (k_{obs}) approaches saturation value. Straight lines are obtained

when $1/K_{obs}$ is plotted versus the reciprocal concentration of substrate, indicating a hyperbolic dependence of K_{obs} on $[S]$. This means that the experimental results can be interpreted using a scheme where free enzyme is assumed to react with substrate to form an intermediate ES prior to the rate-limiting conversion of this intermediate into reduced enzyme E_{red}.



Evaluation of the velocity constants showed that the equilibrium constant (K_1/K_{-1}) for the binding of substrate to the enzyme, exhibited a pH-dependence analogous to the hydrazines.

The results suggest that the non-protonated amine is the reactive form of the substrate and show that the enzymatic pK_a 8,8 group regulating complex formation between hydrazines and benzylamine oxidase also regulates the binding of substrates to the enzyme.

The kinetic parameter K_2 was found to be essentially independent of pH. On the other hand there is a small substituent effect on this parameter, such that electron-donating substituents significantly decrease the magnitude of K_2 . $\log K_2$ varies approximately linearly with the Hammett σ -value for the respective substituents, yielding $\rho = +0,3$.

Isotope effects

Assuming that the prosthetic group in benzylamine oxidase is related to PLP, dissociation of an α -H in the amine substrate would be expected to precede hydrolytic release of the aldehyde product. In order to determine if such a proton dissociation step is kinetically significant, the presence of isotope effects in the catalytic reaction was examined using $[\alpha, \alpha\text{-}^2\text{H}]$ benzylamine as the substrate (paper V). The results showed that substitution of the α -protons in benzylamine by deuterium had no significant effect on the steady-state maximum velocity V , which is consistent with the conclusion that a common reduced enzyme species is formed with all substrates. The K_m -value, however, was found to increase by a factor of about 3, indicating that there is an isotope effect on the reduction process. Direct examination of the reduction process by stopped-flow techniques showed that this isotope effect can be attributed to the reaction step (s) represented by the parameter K_2 in scheme (2). No significant effect was obtained on the equilibrium constant K_1/K_{-1} . The isotope effect on K_2 is sufficiently large to be character-

rized as a primary isotope effect (46). The results provide strong evidence that deprotonation of the α -carbon of benzylamine is ratelimiting for the conversion of the ES-complex into the reduced enzyme species Ered.

Intermediates in the reduction process

During the above studies it was observed that the reduction of benzylamine oxidase by p-methoxybenzylamine under anaerobic conditions, led to biphasic absorbance changes at 470 nm, resulting in a transient increase in absorbance prior to the main decolorization process reflecting formation of the reduced enzyme species. The mechanistic significance of this observation is considered in paper VI.

The results obtained show that the biphasic 470 nm absorbance changes observed in the reaction with p-methoxybenzylamine can be attributed to the transient accumulation of a spectrally modified enzyme intermediate exhibiting maximum absorption at about 360 nm. Kinetic data for the reaction are consistent with the following reaction scheme:



ES is an enzyme-substrate complex with the same spectral properties as free enzyme, ES^* the spectrally modified complex and Ered the reduced enzyme species lacking the 470 nm absorption band. Formation of the intermediate ES^* can also be detected in the reactions with other substrates, provided that the reactions are monitored at wavelengths in the 330-360 nm region. The maximum concentration of ES^* formed with benzylamine is much lower and reached more rapidly than with corresponding concentrations of p-methoxybenzylamine. However, the spectral properties of the intermediate formed with benzylamine, seem to be similar to those of the intermediate formed with p-methoxybenzylamine. When benzylamine is substituted by $[\alpha, \alpha^{-2}H]$ benzylamine as a substrate for the enzyme there is a considerably decreased level of accumulation of ES^* . This effect can be well accounted for by assuming that the rate constant K_1 , in Scheme (3), decreased by a factor of 3 on substitution of the α -protons in benzylamine by deuterium. Consequently, it appears that dissociation of the α -H in the amine substrate precedes formation of the intermediate ES^* .

Intermediates in the Reoxidation process

The data considered in Papers I-VI refer mainly to the mechanism of the partial process of enzyme reduction. A kinetic characterization of the subsequent partial process, leading to reoxidation of the reduced enzyme species, is given in Paper VII.

At high substrate concentrations the reduction process is followed by a slow phase during which the 470 nm absorbance increases slightly until the steady-state level is reached after about 10 S. Time constants for the exponential transient corresponding to this slow phase tend towards a limiting value of $1,3 \text{ s}^{-1}$ at saturating substrate concentrations. Both the rate and amplitude of the recolorization transient increase when the oxygen concentration is raised. These results suggest that the reoxidation process involves at least one oxygen-dependent and one oxygen-independent reaction step, according to the following scheme.

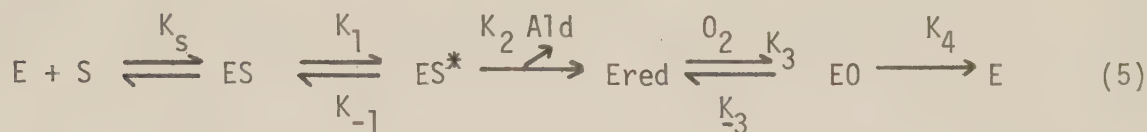


where EO denotes the kinetically significant intermediate formed after the interaction between enzyme and oxygen. Formation of EO leads to complete recolorization of the 470 nm chromophore and the results obtained could be well accounted for under the assumption that Ered is the only kinetically significant enzyme species lacking the 470 nm absorption band. EO also exhibits a difference-absorption band centered around 280 nm in comparison to reduced and free enzyme. This observation suggest that the formation of EO leads to a spectral perturbation of aromatic amino-acid residues in the protein, possibly indicating a conformational change of the enzyme.

Estimates of the rate constants in Scheme (4) were determined by combined steady-state and transient-state kinetic methods. The results show that, under aerobic conditions, the oxygen-independent reaction is the main rate-limiting step in the catalytic process at alkaline pH. At neutral or acid pH the oxygen-dependent reaction becomes the main rate-limiting step. These results indicate that the concentration of oxygen may be a critical factor controlling the catalytic efficiency of the enzyme under physiological conditions.

Discussion

The results summarized in the present thesis establish the following minimal reaction scheme for the catalytic reaction of benzylamine oxidase.



The identity of the enzymatic intermediates involved in this scheme, and the nature of the reaction steps leading to their formation and decomposition, still remains obscure. This is due mainly to the fact that the structure of the prosthetic group in the enzyme has not been unequivocally defined. The evidence presented by Buffoni (36), however, strongly suggests that benzylamine oxidase is a pyridoxal enzyme. The circular dichroism data reported in Paper VI support this idea in showing that the enzyme exhibits an optically active absorption band in the 360 nm region. Such an absorption band is known to be associated with aldimine forms of pyridoxal dependent transaminases and decarboxylases (47). The spectral changes observed in the interaction between benzylamine oxidase and hydrazine derivatives (Paper III) can, similarly, be well understood in terms of hydrazone formation with enzyme-bound pyridoxal phosphate.

Assuming that benzylamine oxidase does contain PLP, it may be considered that the complex ES most likely represents a Schiff-base derivative formed between the amine substrate and enzyme bound PLP. The absence of spectral changes during formation of the initial Schiff-base might be explained by suggesting that the prosthetic group, by analogy to what has been reported for pyridoxal enzymes (47, 48, 49), is attached to native benzylamine oxidase in the form of an intramolecular Schiff-base involving the amino group of a protein residue.

According to what is known about pyridoxal catalysis in general, formation of the reduced enzyme species would be expected to involve a prototropic shift converting the initial Schiff-base between amine and pyridoxal into a Schiff-base between amino aldehyde and pyridoxamine (50).

Such a mechanism is entirely consistent with the results introduced in the present thesis, with hydrolytic release of aldehyde possibly yielding a pyridoxamine.

The existence of pyridoxamine depends on whether ammonia is leaving during the reduction phase or not. Before elucidating this there is no point in discussing the structure of E0.

Although no final conclusion can be drawn, the results provide a good base for the complete elucidation of the reaction mechanism of benzylamine oxidase.

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REFERENCES

1. Zeller, E.A., Stern, R., and Wenk, M. (1940) *Helv. Chim. Acta* 23, 3.
2. Zeller, E.A., "The Enzymes" 2nd ed., Vol. 8, p. 313 (1963).
3. Bandhuin, P., Beautay, H., Rahman-Li, Y., Sellinger, O.Z., Wattiaux, R., Jacques, P., and de Duve, C. (1964) *Biochem. J.* 92, 179.
4. McGeer, P.L. (1971) *Amer. Sci.* 57, 221.
5. Kapeller-Adler, R. (1970) "Amine Oxidase and Methods for Their Study". Wiley (Interscience), New York, p. 191.
6. Blaschko, H., and Bonney, R. (1962) *Proc. Roy. Soc. Ser. B*, 156, 268.
7. Hill, J.M., and Mann, P.J.G. (1964) *Biochem. J.* 91, 171.
8. Buffoni, F., and Blaschko, H. (1964) *Proc. Roy. Soc. Ser. B*, 161, 153.
9. Achee, F.M., Chervenka, C.H., Smith, R.A., and Yasunobu, K. (1968) *Biochemistry*, Vol. 7, No. 12, 4329.
10. Yamada, H., Adachi, O., and Ogata, K. (1965) *Agr. Biol. Chem.* 29, 864.
11. Yamada, H., Kumagai, H., Kawasaki, H., Matsui, H., and Ogata, K (1967) *Biochem. Biophys. Res. Commun.* 29, 723.
12. Hill, J.M. and Mann, P.J.G. (1964) *Biochem. J.* 91, 171.
13. Buffoni, F., Della Corte, L., and Knowles, P.F. (1968) *Biochem. J.* 106, 3077.
14. Yamada, H. and Yasunobu, K.T. (1962) *J. Biol. Chem.*, Vol. 237, 3077.
15. Yamada, H. and Yasunobu, K.T. (1965) *J. Biol. Chem.* 29, 912.
16. Mondovi, B., Rotilio, G., Costa, M.T., Finazzi Agro, A., Chiancone, E., Hanswn, R.E., and Beinert, H. (1967) *J. Biol. Chem.* 242, 1160.

17. Yamasaki, E.F., Swindell, R., and Reed, D.J. (1970) *Biochemistry* 9, 1206.
18. Lindström, A. and Pettersson, G. (1973) *Eur. J. Biochem.* 34, 564.
19. Buttoni, F. and Ignesti, G. (1975) *Biochem. J.* 145, 369.
20. Hücko-Haas, J.E. and Reed, D.J. (1970) *Biochem. Biophys. Res. Commun.* 39, 396.
21. Yamada, H., Suzuki, H., and Ogura, Y. (1972) *Advan. Biochem. Phycho-pharmacol.* 5, 185.
22. Yamada, H., Yasunobu, K.T., Yamano, H., and Mason, H.S. (1963) *Nature* 198, 1092.
23. Buffoni, F. (1966) *Pharmacol. Rev.* 18, 1163.
24. Vänngård, T. (1972) in "Biological Applications of EPR" (Swartz, H.M., Bolton, J., and Borg, D., eds.), Wiley (Interscience) New York, p. 411.
25. Yamada, H., Adachi, O., and Yamano, T. (1969) *Biochim. Biophys. Acta* 191, 751.
26. Goryachenkova, E.V., Scherbatyuk, L.I., and Zamaraev, C.I. (1968) in "Pyridoxal Catalysis: Enzymes and Model Systems" (Snell, E.E. et al., eds.), Wiley, New York, p. 391.
27. Boden, N., Charlton, S.C., Holmes, M.C., and Knowles, P.F. (1973) *Biochem. Soc. Trans.* 1, 1008.
28. Malkin, R. and Malmström, B.G. (1970) *Adv. Enzymol.* 33, 177.
29. Malkin, R. (1975) in "Inorganic biochemistry" (Eichhorn, G.L. ed.), Elsevier, Amsterdam, p. 689.
30. Olsson, B. and Pettersson, G., unpublished results.
31. Lindström, A., Olsson, B., and Pettersson, G. (1974) *Eur. J. Biochem.* 48, 237.

32. Nylén, U. and Szybek, P. (1974) *Acta Chem. Scand.* B28, 1153.
33. Lindström, A., Olsson, B., and Pettersson, G. (1973) *Eur. J. Biochem.* 35, 70.
34. Mondovi, B., Rotilio, G., Finazzi Agro, A., Vallogini, M.P., Malmström, B.G., and Antonini, E. (1969) *FEBS Lett.* 2, 182.
35. Adachi, O. and Yamada, H. (1969) *Agr. Biol. Chem.* 33, 1707.
36. Buttoni, F. and Della Corte, L. (1972) in *Advances in Biochemical Psychopharmacol.* Vol. 5, Raven Press, New York, (Costa, E. and Sandler, M., eds.) p. 133.
37. Kumagai, H., Nagate, T., Yamada, H., and Fukami, H. (1969) *Biochim. Biophys. Acta* 185, 242.
38. Inamasu, M. and Yasunobu, K.T. (1974) *J. Biol. Chem.*, Vol. 249, No. 16, 5265.
39. Hill, J.M. (1967) *Biochem. J.* 104, 1048.
40. Benitez, L.V. and Allison, W.S. (1974) *J. Biol. Chem.* 249, 6234.
41. Neumann, R., Hevey, R., and Abeles, R.H. (1975) *J. Biol. Chem.* 250, No. 16, 6362.
42. Taylor, C.E., Taylor, R.S., Rasmussen, C., and Knowles, P.F. (1972) *Biochem. J.* 130, 713.
43. Oi, S., Inamasu, M., and Yasunobu, K.T. (1970) *Biochemistry* 9, 3378.
44. Reed, D.J. and Swindell, R. (1969) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* 28, 891.
45. Finazzi Agro, A., Rotilio, G., Costa, M.T., and Mondovi, B. (1969) *FEBS Lett.* 4, 31.
46. Saunders, W.H. (1974) in *Investigation of Rates and Mechanisms of Reactions* (Lewis, E.S., ed.) 3rd edn., Interscience, London, p. 211.

47. Torchinsky, Y.M., Malakhova, E.A., Livanova, N.B., and Pikhelgas, V.Y. (1968) in Pyridoxal Catalysis: Enzymes and Model Systems (Snell, E.E., Braunstein, A.E., Severin, E.S., and Torchinsky, Y.M., eds.) Interscience, New York, p. 269.
48. Sizer, I.W. and Jenkins, W.T. (1963) in Chemical and Biological Aspects of Pyridoxal Catalysis (Snell, E.E., Fasella, P.M., Braunstein, A., and Rossi, Fanelli, A., eds.) Pergamon, Oxford, p. 123.
49. Wilson, M.E., Novogrodsky, A., and Meister, A. (1968) in Pyridoxal Catalysis: Enzymes and Model Systems (Snell, E.E., Braunstein, E.A., Severin, E.S., and Torchinsky, Y.M., eds.) Interscience, p. 425.
50. Jencks, W.P. (1969) in Catalysis in Chemistry and Enzymology, Mc Graw-Hill, London, p. 133.

